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Research Report

Repeated blast exposure alters open field behavior recorded under low illumination

Emerald Huang[a], *Mai Ngo*[a], *Stuart Yee*[a], *Laura Held*[b], *Keith Norman*[c], *A.M. Erika Scremin*[b,d], *Oscar Scremin*[c,e,*]

[a]UCLA/VA Multi-campus Residency Program, Los Angeles, CA, USA
[b]PM&R, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA, USA
[c]Research Services, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA, USA
[d]Medicine Department, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
[e]Physiology Department, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

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ABSTRACT

Blast-induced traumatic brain injury (bTBI) can have devastating behavioral consequences. This study was designed to evaluate the behavioral consequences of single or repeated bTBI, as evaluated by an open field (OF) test conducted in near-darkness to avoid confounding effects of illumination and photophobia. Sprague-Dawley rats under isoflurane anesthesia were exposed to a series of 3 sub-lethal blasts into a compressed air-driven blast chamber separated by 2 week intervals ($n=11$). Sham controls received anesthesia but without blast exposure ($n=11$). OF tests were performed 1 or 7 days after each blast using a computerized video tracking system in near-darkness to monitor spontaneous activity. Spatial and temporal variables calculated for both blast and sham groups were: Distance moved (cm) and time (s) spent in the center or periphery zones of the field, total distance traveled, speed in center and periphery zones, rearing events and non-linear regressions of distance moved and rearing events on time. Results showed that the sham group expressed the expected decrease (habituation) in total distance walked, and distance walked as well as speed in center and periphery in successive exposures to the OF while the blast group did not, a sign of impaired learning. The blast group also walked more and faster and demonstrated more rearing behavior, both considered OF signs of anxiety. These results indicate that OF outcomes of bTBI in animals have resemblance to alterations observed in human subjects with this condition and might be useful in evaluating the response of behavioral outcomes of bTBI to experimental treatments.

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1. Introduction

Blast-related traumatic brain injury (bTBI) is a more frequently encountered injury in current conflicts. While there are no systematic evaluations of health consequences for survivors of blasts in the civilian population in areas of conflict, according to the "Defense and Veterans Brain Injury Center," 244,217 TBIs have been identified up to the

*Corresponding author at: Veterans Affairs Greater Los Angeles Healthcare System, Department of Research, 11301 Wilshire Blvd., Bldg. 115, Room 319, Los Angeles, CA 90073, United States. Fax: +1 310 268 4209.
E-mail address: oscremin@ucla.edu (O. Scremin).

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first quarter of 2012 amongst all US armed forces. 76.8% of those TBI cases are considered mild and of those, approximately 50% are due to blast injuries (Defense and Veterans Brain Injury Center and Armed Forces Health Surveillance Center, 2011).

TBI and PTSD are considered signature injuries of the current war in Iraq and Afghanistan given the increased use of conventional and improvised explosive devices (Tanielan and Jaycox, 2008; Spelman et al., 2012). Even for mild TBI, defined as a confused or disoriented state which lasts less than 24 h; loss of consciousness for up to 30 min; memory loss lasting less than 24 h; and structural brain imaging (MRI or CT scan) yielding normal results, the functional deficits can be severe and long-lasting.

Animal models have been used to simulate blast TBI. However, the validation of the models requires comparison to human data in order to demonstrate that they reproduce one or more relevant features of human injury. Clinically, anxiety and depression are very prevalent mental health conditions in human subjects with mild TBI (Spelman et al., 2012; Moore et al., 2006; Mooney and Speed, 2001). Rodent models for these conditions have been proposed. Among them, unconditioned paradigms including avoidance of exposed, brightly lit spaces (elevated T or plus maze, light-dark box and open fields with bright illumination) have been widely used in rats and mice (Sartori et al., 2011). Preference of the covered arms of the first, the dark sector of the second and avoidance of the center of or freezing behavior in the third have been taken by many authors as "anxiety-like" behaviors (Cryan and Sweeney, 2011). However, in the case of TBI, photophobia is of common occurrence in humans (Tanielan and Jaycox, 2008; Spelman et al., 2012; Stovner et al., 2009; Kapoor and Ciuffreda, 2002; Bohnen et al., 1991) and possibly also in experimental animals. Moreover, light inhibits ambulation in rodents (Livesey and Egger, 1970; Valle, 1970; Blizard, 1971) and albinism, present in most of the rat strains used as TBI models, increases the light sensitivity even more (Dixon and DeFries, 1968). Thus, in order to examine the open field behavior of rats subjected to blast brain trauma, illumination should be minimized to realize the full ambulatory capacity of the animals and prevent the confounding effect of photophobia.

The present experiments were conducted in near-darkness with detection of movement by use of an infrared light source and an infrared-sensitive camera. In addition, and to model a common condition encountered in the field, multiple blasts, followed by OF tests in each case, were induced. This protocol also evaluated the magnitude of the inter-session habituation, i.e. a decrease in exploratory activity on successive exposures to the novel environment, that is considered a primitive form of learning, and its absence as an evidence of "forgetfulness" of the characteristics of the field environment, requiring renewed exploration at every exposure to it, (Russell, 1982a) that could model the memory problems reported in subjects with mild TBI (Terrio et al., 2009).

The more common blast injury models make use of blast tubes, which simulate field conditions in the open, showing a pressure profile similar to a Friedländer curve. In the present application, we have used a closed receiving chamber with a leak, providing a complex pressure profile to more realistically reproduce conditions encountered for blasts in urban environments (Cernak et al., 2011).

Soldiers are subjected to repeated blasts in training and in theater; however, there are limited studies on the neurological effects of repeated blast exposures. In second-impact syndrome as defined for sports, patients suffer an initial mild concussive head injury that is hypothesized to induce cerebral autoregulatory failure. Then, a second impact might produce a systemic stress-induced catecholamine surge and rapidly elevated blood pressure that results in devastating acute and massive brain swelling (Wetjen et al., 2010). A discrete return-to-play guideline has been established for all brain-injured athletes (Aubry et al., 2002). Limited studies looking at physiologic and anatomic changes from tightly coupled repeated bTBI in mice noted that neurodegeneration was found in various parts of the brain. Motor performance is also compromised in these mice (Wang et al., 2011).

Since 90% of rat genes possess strict orthologues in the human genome, they serve as appropriate subjects for the preclinical stage of research and for the sake of safety and time efficiency (Gibbs and Weinstock, 2004). This species has been selected to the present study carried out with a novel chamber device generating a blast of controlled intensity adjusted to induce a functional deficit without lethality. A repeated series of blast events was used to simulate field conditions with multiple exposures.

The main objective of this study was to determine if a simple procedure, the open field test, could demonstrate evidence of markers of anxiety or impaired spatial learning that are common to human cases of mild bTBI. A secondary objective was to determine which of the variables defined in this test showed the better power to differentiate between injured and control subjects.

2. Results

Peak pressures recorded inside the blast chamber were reproducible and no significant differences existed among the 1st, 2nd and 3rd blasts (Table 1). The pressure transient during the blast, measured with a piezoelectric sensor placed next to the animal head, oriented towards the mylar membrane indicated a fast (<2 ms) rise time, followed by a biphasic drop and a slightly negative pressure afterwards (Fig. 2).

Rats in the blast group walked significantly more in total distance (sum of distance traveled in the center and periphery of the field) than rats in the sham group ($P=0.035$) during the 20 min trial (Fig. 3 and Table 2). Animals in the SHAM group decreased their total distance traveled with repeated

Table 1 – Peak pressures (Mean and SD, kPa) recorded inside the blast chamber in all experiments. There were no significant differences among blast pressure means.

	Blast 1 (kPa)	Blast 2 (kPa)	Blast 3 (kPa)
Mean	175.0	174.2	163.9
SD	23.9	37.5	23.6

Table 2 – Significance and power of ANOVA F-ratio in all open field variables for the factors: treatment, blast/sham number, and days post blast or sham.

Variable	Treatment (Blast vs sham)		Blast/Sham number (1, 2, or 3)		Days post (1 vs 7)	
	P value	Power	P value	Power	P value	Power
Time on center	0.187	0.260	0.431	0.191	0.115	0.350
Time on periphery	0.163	0.285	0.573	0.140	0.124	0.336
Distance walked in center	0.556	0.090	0.734	0.098	0.156	0.293
Distance walked in periphery	0.002*	0.896	0.036*	0.632	0.045*	0.520
Total distance walked	0.035*	0.565	0.176	0.362	0.906	0.052
Walking speed in center	<0.001*	0.991	0.096	0.473	0.332	0.161
Walking speed in periphery	0.039*	0.547	0.517	0.158	0.932	0.051
Time on periphery over time on center	0.281	0.188	0.570	0.141	0.599	0.082
Total distance walked on periphery over total distance walked on center	0.169	0.279	0.843	0.076	0.300	0.178
Rearings	0.017*	0.669	<0.001*	1.000	0.003*	0.845

* Indicates statistical significance.

exposures to the OF (ANOVA $P=0.034$) while animals in the blast group did not (Fig. 3).

The distance walked in the center was not significantly different between groups or between blast or sham repeated events (Fig. 4 top panel) while distance walked in the periphery was greater for the blast group ($P=0.002$) and showed a progressive decrease with successive increases in exposures to the OF in animals on the sham group ($P=0.0043$) while animals in the blast group did not (Fig. 4, bottom panel).

The blast group walked significantly faster than the sham group in the center and periphery of the OF ($P<0.001$ and $P=0.039$ respectively) (Fig. 5 and Table 2). The sham group progressively decreased their walking speed in both regions with successive exposures to the OF while the blast group did not.

The blast group had a significantly greater number of rearings, (the times that the rat stood on its hind legs) than the sham group ($P=0.017$), and as described below, both groups also differed in the behavior of this variable with repeated exposures to the OF.

Non-linear regression of distance moved over time during the OF test showed a decrease over time that was adequately modeled by a single exponential decay with a plateau (Table 3 and Fig. 6). Comparisons between data sets using the extra sum of squares F-test indicated significant differences of the global parameter set between sham and blast groups at all the successive events (Table 3). In the sham group, a progressive decrease in the final plateau value and acceleration of the rate of decay of distance moved was observed. Comparison of the global fit parameters between the first and last set of events in the sham group yielded a highly significant difference (Table 3). In contrast, no difference was found in a similar comparison for the blast group. Both groups also differed significantly when the last events were compared, indicating a significantly higher final plateau value for the blast group (Table 3 and Fig. 6).

Non-linear regression analysis of rearing events over time in the sham group indicated lack of time dependence of this variable in the first OF event but a statistically significant dependence in the third event, with goodness of fit (Rsquare) of 0.27, $P=0.023$. In the blast group, a significant regression of rearing on time was found for the first and last events (goodness of fit (Rsquares) 0.26, $P=0.025$ and 0.48, $P<0.001$ respectively) but neither the global nor the individual fit parameters differed significanlty between both events. The global fit parameters for the third sham and blast events differed significantly ($P=0.0017$) (Fig. 7). There was considerably more variance in rearing events than in distance moved, as a comparison of the Rsquare values consigned above with those shown in Table 3 and the scatter around fit curves in Figs. 6 and 7 clearly demonstrate.

Analysis of differences between days 1 and 7 post sham or blast events did not show statistical significance for any of the variables, except for number of rearing events in the blast group on the third blast event (Day $1=11\pm6.8$, Day $7=77.8\pm10.3$).

3. Discussion

Our study indicates good tolerability of animals to the blast procedure and reproducibility of the pressure transients generated in the blast-simulating device. The open field test, a simple behavioral task that may allow high throughput screening of blast-injured animals, provided several measures of elements found in humans with mild bTBI, such as hyperactivity, depression, anxiety and alterations in learning and retention of spatial information in novel environments.

One of the objectives of the study was to determine which variables in the OF evaluations had better statistical power for this particular population of experimental subjects. It was found that walking speed in the center of the field, distance walked in the periphery of the field, rearings, and total distance walked, fulfilled the requirement of acceptable power for the evaluation of outcomes in this experimental paradigm as is shown in Table 2.

Non-linear regression analysis of activity over time in the novel environment provided information on the initial level, the rate constant of decay and the final plateau level of

Table 3 – Best fit parameters of non-linear regressions of distance moved on time. S1-3: Sham Group Sessions 1–3; B1-3: Blast Group Sessions 1–3; Y_0: Initial value; k: rate constant; S1-3 vs B1-3: Statistical significance of comparisons between Sham and Blast groups curves; S1 vs S3 and B1 vs B3: Statistical significance of comparisons between the first and last sessions of the Sham or Blast Groups respectively.

	S1	S2	S3		S1 vs S3
Y_0	324.3±23.0	450.1±36.6	536.9±76.7	Yo	P=0.0002*
Plateau	151.1±15.4	136.7±5.6	91.69±10.4	Plateau	P=0.0037*
k	0.17±0.05	0.36±0.06	0.48±0.11	*k*	P=0.0739
Rsquare	0.84	0.92	0.95	ALL	P< 0.0001*
	B1	**B2**	**B3**		**B1 vs B3**
Y_0	595.3±48.7	518.3±65.9	488.3±76.7	Yo	P=0.3111
Plateau	151.1±7.37	169.7±7.1	164.4±10.4	Plateau	P=0.744
k	0.41±0.06	0.52±0.12	0.36±0.11	*k*	P=0.4102
Rsquare	0.93	0.85	0.80	ALL	P=0.2678
	S1 vs B1	**S2 vs B2**	**S3 vs B3**		
ALL	P<0.0001*	P=0.0201*	P<0.0001*		
Y_0	P<0.0001*	P=0.4429	P=0.6147		
k	P=0.021*	P=0.3071	P=0.4612		
Plateau	P=0.9973	P=0.0069*	P=0.004*		

* Indicates statistical significance.

distance moved and rearing events for each OF session. Preference by the animals for the center or periphery of the OF zones did not show a dependence on time within the test. The goodness of fit of the data to the model was considerably better for distance moved than for rearing activity. Moreover, the rearing activity of sham control animals in the first OF session was not dependent at all on time within the OF test. Thus, the regression of distance moved on time provided the best dynamical assessment of open field behavior. The lack of between session habituation of the blast group was indicated for this variable by a higher final plateau level of distance moved and the lack of differences between rate constant and plateau parameters in a comparison between the last and first OF session. In contrast, an increase in rate constant and a decrease in plateau level in the same comparison, both indicative of between sessions habituation was found in the sham group. As a corollary of these findings, integration of distance moved over the entire test period, indicated the expected decrease in distance. The conclusion is that the repeated blasts brought about a deficit of within and between session habituation of distance moved. Habituation in the OF is considered a primitive form of learning, and the lack of it, a failure of memory of past experiences (Russell, 1982b). This may model some of the cognitive deficits found in human cases of blast injury.

The hyperactivity condition manifested by animals in the blast group (enhanced distance walked and speed and increased rearing) is in line with previous observations in animals subjected to blunt force (Pandey et al., 2009) or lateral fluid percussion brain trauma (Li et al., 2006) and with similar findings in bulbectomized rats (Song and Leonard, 2005). Hyperactivity in these animals may model hyperactivity observed commonly after TBI, as manifested by post-traumatic impulsiveness, agitation, desinhibition, restlessness and akathisia. Given that a deficit in central cholinergic function is known to lead to hyperactivity (Russell et al., 1990), and the well documented deficit of this system in experimental TBI, it is tempting to speculate that a decrease in acetylcholine turnover or alterations in the number or affinity of cholinergic receptors might underlie the observed changes.

A study with the mice model where the animals had sustained a single blast injury, has indicated a decline in open field activity, characterized by both less distance traveled and decreased speed (Cernak et al., 2011). However, the study was conducted under high illumination of the test arena (550 lx), a condition that inhibits locomotion in rodents, a phenomenon that could be additionally enhanced by photophobia, one of the common complaints of blast TBI victims probably shared by experimental animals with the same condition (Theeler et al., 2012). The present work was conducted in near-darkness (0.1 lx) to avoid the potential complication of photophobia and to allow the animals to display their maximal exploratory activity without the physiological inhibition induced by intense light.

It is interesting to note that the differences between groups were not always present after the first session, highlighting the importance of testing animals after repeated sessions of blast injury rather than after a single session. These results represent the first steps in validating a repeated bTBI rat model. For future research, additional cognitive and motor tests, for example, the water maze, rotarod, and startle response, should be performed to evaluate the specific deficits that cause behavioral alterations. It is also key to define the critical number of repeated blast exposures that causes long-lasting damage. Finally, outcome measures presented could be used to evaluate interventions that may improve or reverse the pathology associated with multiple blast exposures.

Blast TBIs differ from other forms of TBI in their biomechanism and neuropathology. The pathophysiology of bTBI is complex, commonly grouped by injuries resulting from different mechanical aspects of the blast phenomenon: primary bTBI is from the shockwave, secondary from shrapnel,

tertiary from the blast wind, and quaternary from chemical or thermal burns (DePalma et al., 2005). Most unique is the primary blast injury induced by the direct shockwave transmission through the skull and the respiratory airways and vascular system. Even though *in-vivo* experiments have shown that significant intracranial pressure can transmit through the brain during a blast, the biological mechanism of shockwave transmission and how it causes cellular damage is largely unknown (Chen et al., 2009). It is even less clear how bTBI differs in its neurobehavioral abnormalities from the better studied cortical contusion induced TBIs.

In conclusion, this study has shown that the sham group expressed the expected habituation in distance walked, walking speed and rearing between and within sessions in successive exposures to the OF while the blast group did not, a sign of impaired learning. The blast group also walked faster and demonstrated more rearing behavior, both considered OF signs of anxiety. The study has also provided a method, non-linear regression of distance moved on time, to give precision to the study of within session adaptation. Blast-injured animals habituated less than sham controls within a session, while maintaining the same initial level and rate of decay of activity over time, but lost completely the habituation between sessions, indicative of an inability to recall the characteristics of the novel environment over the two weeks span over sessions. The number of rearing events and the walking speed were higher in the animals that received blasts, both associated with anxiety as expressed in this test. These results indicate that OF outcomes of bTBI in animals have resemblance to alterations observed in human subjects with this condition and might be useful in evaluating the response of behavioral outcomes of bTBI to experimental treatments.

4. Experimental procedure

4.1. *Care of experimental animals and anesthetic procedures*

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Greater Los Angeles Healthcare System in compliance with the guidelines for animal experimentation of the National Institutes of Health. We used Sprague-Dawley male rats weighing 215–300 g as our subjects. Animals were housed in pairs in standard cages on a 12 h light/dark cycle at constant room temperature (21 °C) with free access to food and water. Animals were randomly assigned to either a blast group ($n=11$) or sham group ($n=11$).

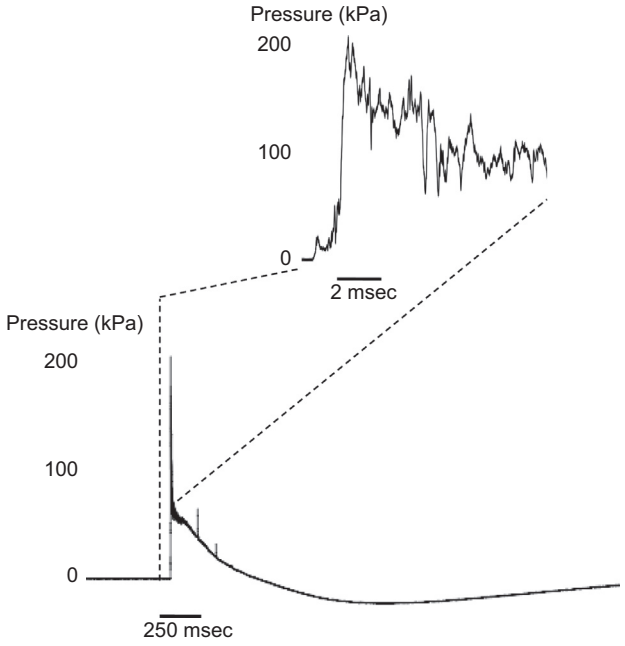

Fig. 2 – Record of pressure transient inside the blast chamber. A piezoelectric sensor (Model 102B16 Dynamic Pressure Sensor, Model 482A21, PCB Piezotronics, Depew, NY) and connected to a Piezotronics Sensor Signal Conditioner and AD Instruments Power lab digitizer (AD Instruments, Colorado Springs, CO) was placed next to the animal head, oriented towards the mylar membrane. The peak pressure (faster time-base inset) is reached at 1.4 ms from onset of the blast and this is followed by a biphasic drop and a slightly negative pressure afterwards.

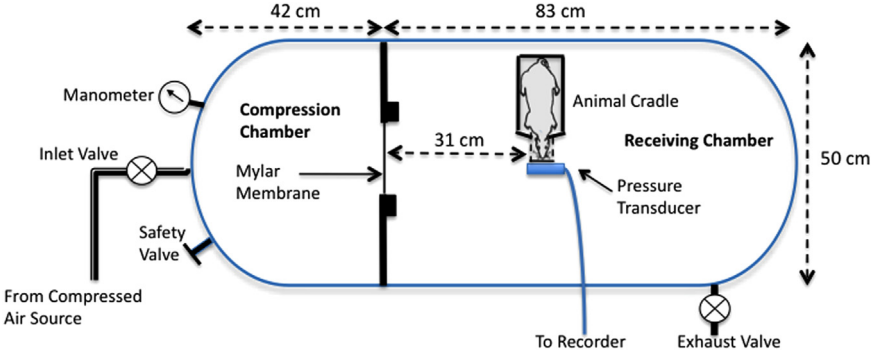

Fig. 1 – Schematic diagram of the compressed air-driven blast chamber used to induce blast trauma in the rats. The compression and receiving chambers were joined securely in the position shown in the diagram during operation but could be separated and pivoted to introduce the animal in the cradle. A typical record of the pressure transient recorded during a blast is shown in Fig. 2.

4.2. *Blast trauma model*

A compressed air-driven blast chamber was used for the blast overpressure of the rats (Custom Technologies Equipment, Ames, Iowa). This device consisted of a compression chamber that was pressurized from a source of compressed air to a maximum of 40 psi. A circular opening fitted with a mylar membrane separated this chamber from another in which animal was placed in a plastic holder that protected the body but left the head exposed with its antero-posterior axis perpendicular to the direction of blast shock waves (Fig. 1). A continually building pressure was generated on the compression chamber to a certain air pressure until the mylar film separating the compression chamber and the receiving chamber ruptured, sending a pressure wave towards the exposed head of the rat. Peak pressures recorded in all experiments are shown in Table 1 and an example of the pressure record over time obtained with a Model 102B16 Dynamic Pressure Sensor, Model 482A21 Sensor Signal Conditioner (PCB Piezotronics, Depew, NY) and AD Instruments Power Lab digitizer (AD Instruments, Colorado Springs, CO) is shown in Fig. 2. These pressure levels have been shown in other experimental studies to cause mild to severe injury (Koliatsos et al., 2011).

Prior to introduction to the blast chamber, animals were anesthetized with 3.5% isoflurane in 30% O_2, balance N_2, for 6 min by a mask with a scavenging system preventing flow of the anesthetic into the environment, while immobilized inside a plastic cone (Decapicone, Braintree Scientific, Inc. Braintree, MA) with the head exposed. They were then positioned into a rigid polycarbonate cylinder with a screw cap on one end that secured the plastic envelope in order to prevent displacement of the animal body inside the tube. The

Fig. 3 – Total distance walked during the open field session (Mean ± SE) was greater in the blast group than in the sham control group ($P=0.035$).

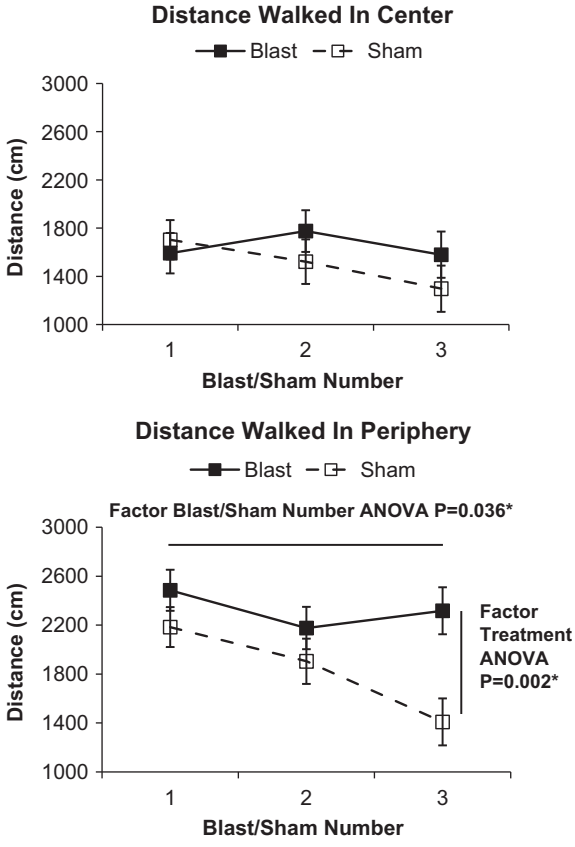

Fig. 4 – Distance walked in the periphery of the field (Mean ± SE, bottom panel) was greater for the blast group (ANOVA $P=0.002$) and showed a difference over number of sham or blast events (ANOVA $P=0.036$). Distance walked in the center of the field did not show significant differences across groups or number of sham or blast events (top panel).

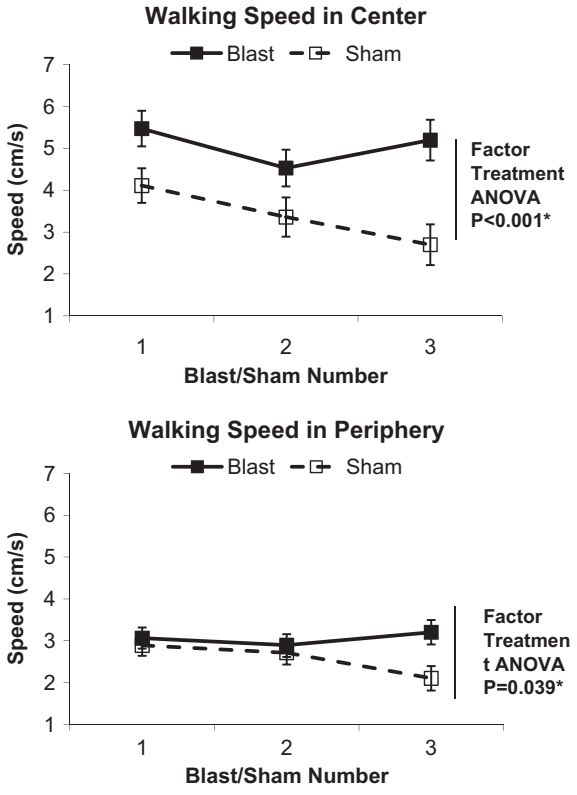

Fig. 5 – Walking speeds (Mean ± SE) in the center (top panel) and periphery (bottom panel) of the field were faster in the blast group than in the sham group ($P<0.001$ and $P=0.039$ respectively).

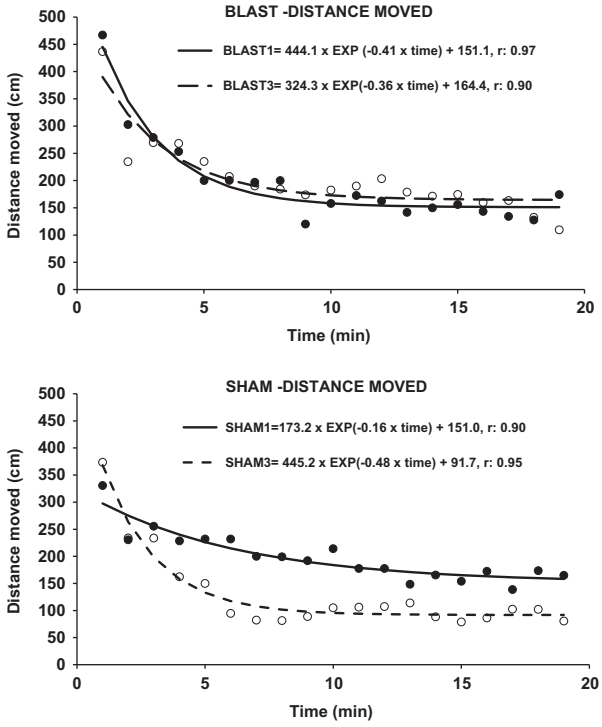

Fig. 6 – Non-linear regression of distance moved (averaged every minute) on time during the open field test for the blast (top) and sham (bottom) groups. Data from the first (solid line) and third (dashed line) events for the blast or sham groups are shown. The Marquardt algorithm was used to fit the data to a model of single exponential decay with a plateau described in the text. Fit parameters and significance of differences between data sets are shown in Table 3.

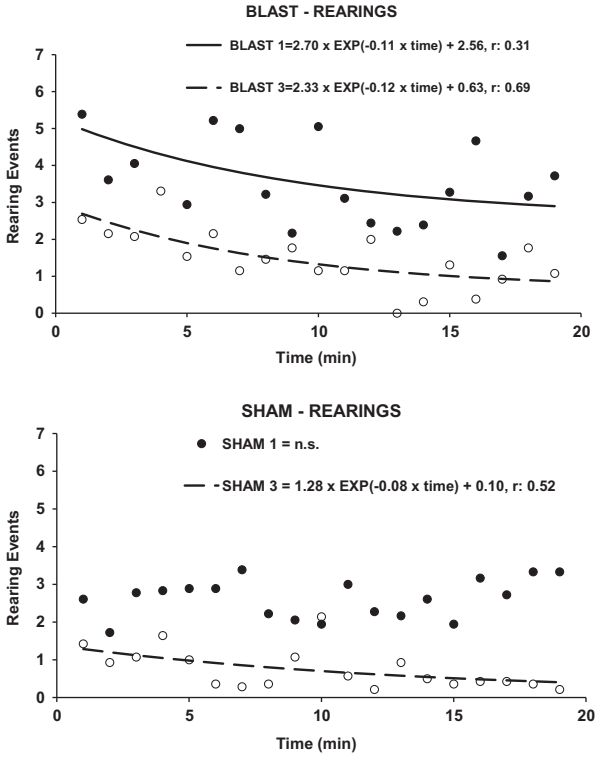

Fig. 7 – Non-linear regression of number of rearing events (averaged every minute) on time during the open field test for the blast (top) and sham (bottom) groups. Data from the first (solid line) and third (dashed line) events for the blast or sham groups are shown.. The Marquardt algorithm was used to fit the data to a model of single exponential decay with a plateau described in the text. There was no time dependence of rearing events on time for the first sham event. The curves for the third sham and blast events differed significantly ($P=0.0017$) but the ones of the first and last blast events did not.

other end of the tube was open, exposing the head of the animal. Movement of the head was limited by a grid of polycarbonate bars. Animals in the Blast group received a blast after the chamber was closed. Immediately after the blast was produced the chamber was decompressed and the animal removed from it. The entire blast procedure lasted between 1 and 1.5 min and the animals were still anesthetized (no withdrawal to hindpaw pinch) when removed from the chamber. Animals in the sham group were anesthetized and introduced into the polycarbonate cylinder but no blast was delivered.

A series of 3 blast or sham exposures were conducted 14 days apart from each.

4.3. *Open field exploration*

The OF test was conducted at 24 h and again at 7 days after each blast or sham intervention. Spontaneous activity was measured in a circular open field chamber, a tank 120 cm in diameter and walls 40 cm high painted black to maximize contrast between animal and background for the purpose of facilitating video tracking. The trajectory of the animals was recorded and digitized with a video tracking system (Ethovision, Noldus Inc., The Netherlands) in near-darkness using infrared video monitoring. All lights in a windowless experimental room were turned off, with the exception of the screen of a computer monitor situated 3 m away from the field. Illuminance on the walking surface of the field was measured with a luxometer (Omega Engineering, Inc. model HHLM-2, Stamford, CT, USA) and found to be 0.1 lx. The field was illuminated with an infrared LED source of 850 nm wavelength (LIR CA-88, Vicon Inc. Hauppage, N.Y.). Rats spectral sensitivity is well outside of this wavelength (Birch and Jacobs, 1975).

The animal trajectories during 20 min trials were analyzed with a Matlab® script that segmented the field on "center" and "periphery" zones and calculated a number of variables described below. The surface area of these two virtual zones was designed to be the same to avoid the confounding effect of zone area on the analysis of zone preference.

In order to determine the activity levels and zone preference of the rats as an indication of their exploratory behavior the following variables were averaged over the entire 20 min test period: time and total distance walked on center and periphery of field, average speed on both zones, total distance walked on field, and the number of rearings. Time dependence of distance moved, rearings and zone

preference along the 20 min of the test were quantified by averaging those variables at 1 min intervals and then computing the regression of the chosen variable on time using a single exponential decay with a plateau as defined in the following equation:

$$Y = (Y_0 - \text{Plateau}) * \text{EXP}(-k * \text{time}) + \text{Plateau}$$

where Y_0=initial value of the variable, k=rate constant (1/time), and plateau=final value of the variable.

4.4. *Statistical analysis*

Statistical analysis was performed using NCSS software (NCSS, Kaysville, Utah 2007). Factorial analysis of variance (ANOVA) was used with factors intervention (blast or sham) blast/sham repetition (1, 2, or 3) and days after blast (1 or 7). If the ANOVA F-ratio was statistically significant ($P<0.05$), multiple comparisons were performed with the Tukey–Kramer test. Statistical significance was set at $P<0.05$ throughout.

Data of mean variable values for every minute of the test were fit to the single exponential decay model with a plateau described above. Goodness of fit was established by the value of R-square and significance of differences between fit parameters of different data sets were calculated by the extra sum of squares F-test using Prism-5 (Version 5.04) software (GraphPad Software, Inc. La Jolla California).

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